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Chronic vascular risk factors (cholesterol, homocysteine, ethanol) impair spatial memory, decline cholinergic neurons and induce blood–brain barrier leakage in rats in vivo

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ABSTRACT

Epidemiological studies show that vascular risk factors (e.g. atherosclerosis, diabetes, homocysteine, hypertension or cholesterol) may play a role in the development of Alzheimer's disease. Animal models may help to discover the role of vascular risk factors on cognition. In the present project we treated male Sprague Dawley rats with a diet containing homocysteine (*hyperhomocysteinemia*) or cholesterol (*hypercholesterolemia*) for 5 months or exposed the rats to *ethanol* (20% in drinking water) or a combination of *cholesterol + ethanol* (*mix*) for 12 months. Our experiments show that all 3 treatments (homocysteine, cholesterol, ethanol) declined spatial memory in the 8-arm radial maze, reduced the number of cholinergic neurons and induced blood–brain barrier leakage in the cortex. Rats treated with cholesterol also displayed markedly enhanced inflammation in the cortex. Levels of amyloid precursor protein, beta-amyloid$_{(1-42)}$, as well as tau and phospho-tau 181 were significantly enhanced in the cortex of cholesterol-fed rats. A combination of ethanol and cholesterol did not further potentiate the effects on spatial memory, cholinergic neurons and blood–brain barrier leakage. The data suggest that chronic mild vascular risk factors over months induce small lesions of the brain capillaries in the cortex, which may contribute to the development of vascular dementia or also Alzheimer's disease.

1. Introduction

1.1. Alzheimer's disease (AD)

AD is characterized by cerebrovascular damage and neuronal dysfunction leading to progressive cognitive decline. The hallmark pathologies include beta-amyloid (Aβ) deposition in brain (plaques) and vessels (Aβ-angiopathy), neurofibrillary tangles containing hyperphosphorylated tau, blood–brain barrier leakage and increased microglial reactivity as well as inflammatory processes. Blood–brain barrier dysfunction is associated with a reduction of cerebral blood flow, hypoxia and accumulation of neurotoxic molecules in brain parenchyma [1,2]. The amyloid hypothesis suggests that the accumulation of Aβ is the most important cascade for the development of AD [3]. Aβ-peptides (40, 42 or 43 amino acids) originate from the membrane-associated amyloid precursor protein (APP) by cleavage with α-, β- and γ-secretases and a dysbalance in Aβ-production and clearance may trigger neurodegeneration [3]. Vascular risk factors (e.g. cholesterol, homocysteine or ethanol) over long periods may cause such a dysfunctional Aβ-clearance as well as blood–brain barrier impairment and possibly initiate cerebrovascular dysfunction or inflammatory processes leading to the development of AD [4–8].

1.2. Vascular dementia (vaD)

AD and vaD share many risk factors suggesting a related pathogenesis [9]. The differentiation of AD from vaD is very difficult, because many symptoms of both diseases are overlapping. Thus, the differentiation is based on evidence of cerebrovascular dysfunction in vaD [8]. Indeed, cerebral vessel pathology results in blood–brain barrier leakage and such multiple cortical infarcts (silent strokes) may play a major role in development of vaD. This is accompanied by ischemic changes with cerebral hypoperfusion and oxidative stress. Clinical symptoms are multifaceted depending on location and size of the stroke lesions which are often asymptomatic for a long period. Vascular risk factors, such as cholesterol, homocysteine and ethanol may play an important role in the development of vaD [10] which is suggested by the fact that vaD is potentially preventable by life style modification and counteracting vascular risk factors [11].

1.2.1. The cerebrovascular system

In contrast to leaky vessels in peripheral organs [12], the blood–brain barrier restricts entry of polar molecules into the brain. Nutrients such as vitamins, glucose, and amino acids cross the blood–brain barrier

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by using specific transporters [13]. Peptides in general poorly cross the blood–brain barrier [14,15], but they can be transported into the brain via specific receptors expressed in brain endothelium under physiological or pathological conditions [16,17]. Intact neurovascular functions are necessary for proper neuronal structure and function. Thus, pericyte deficiency leads to microvascular degeneration and brain accumulation of toxic substances (e.g. Aβ) preceding neuronal degenerative changes, learning and memory impairment and neuroinflammatory response [18,19].

1.3. Methodological aspects

1.3.1. Treatment of rats

Male Sprague Dawley rats (aged 6 months) were housed at the Animal Department of the Medical University Innsbruck and had free access to food and tap water with a 12/12 h light–dark circle. All animal experiments were approved by the Austrian Ministry of Science. The diet of the control animals contained following ingredients: 450 g/kg cornstarch, 140 g/kg casein, 155 g/kg maltodextrin, 100 g/kg sucrose, 40 g/kg soybean oil, 50 g/kg fiber, 35 g/kg mineral mix, 1.8 g/kg L-cystine, 1.4 g/kg choline chloride, 0.008 g/kg butylhydroxytoluol, 10 g/kg vitamin mix (without folic acid), 1 g/kg chocolate aroma and 0.002 g/kg folic acid (Ssniff special diet GmbH; Soest Germany). The animals of the cholesterol group were fed for 5 months (n = 10) or 12 months (n = 12) with additional 50 g/kg cholesterol [20]. The animals of the homocysteine group were fed with additional 3 g / kg dl-homocysteine for 5 months (n = 9) or 15 months (n = 10) [21]. The animals of the ethanol group were treated with 20% ethanol in drinking water ad libitum for 12 months (n = 12) [22]. The animals of the cholesterol-homocysteine mix group were treated with a combined diet of 3 g / kg dl-homocysteine and 5% cholesterol for 5 months and the animals of the cholesterol ethanol mix group (n = 12) were fed with a diet containing 5% cholesterol and 20% ethanol in drinking water [21,22].

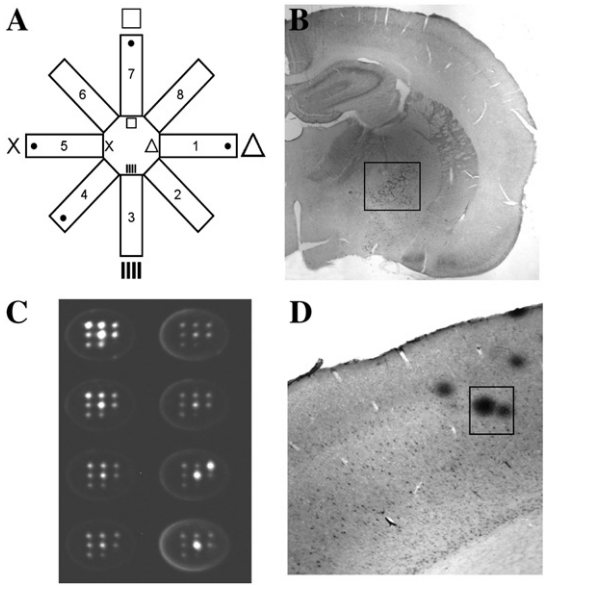

Fig. 1. (A) Spatial learning and long-term memory performance was assessed in the partially baited eight-arm radial maze. For spatial navigation small high contrast visual cues (triangle, vertical bars, cross and squares) were placed above the doors of four arms and on the corresponding walls. Four arms were baited with food pellets and the trial ended when all baits were found or after 10 min exceeded. (B) Rat brains were sectioned into 60 μm slices which were stained for the enzyme choline-acetyltransferase (ChAT). The number of ChAT-positive neurons located in the nucleus basalis of Meynert (small insert) was counted under the microscope. (C) Inflammatory markers were measured by multiplex ELISA. For the ELISA cortex extracts were added to pre-spotted plates, immune-detection was performed and the luminescent signal was evaluated by the Searchlight imaging and analysis system. (D) Blood–brain barrier leakage was assessed by using immunohistochemistry against rat immunoglobulin G (IgG). Rat IgG-positive spots (small insert) in the cortex were counted under the microscope at a 40× magnification.
B, adapted from Pirchl et al. [21]); D, adapted from Ehrlich et al. [22].

1.3.2. Cognition in the partially baited eight-arm radial maze

Spatial learning and long-term memory performance was assessed in the partially baited eight-arm radial maze (Fig. 1A), a well-established method to explore learning and memory in a controlled environment, as recently described by us in detail [21]. The maze consists of eight identical arms with side panels and sunk-in-food cups at the end radiating from a circular platform. For spatial navigation small high contrast visual cues (triangle, vertical bars, cross and squares) were placed above the doors of four arms and on the corresponding walls. Four arms were baited with food pellets (chocolate cereals) and the trial ended when all baits were found or after 10 min exceeded. The task for the animals was to find all baits in respective arms without visiting unbaited arms. The animals were restricted to food before the learning sessions were started to increase motivation. In a shaping session the rats were habituated to the testing procedures to decrease stress. In five training sessions (with each five trials) the animals had to learn the task. After three weeks of the last session the retention (five trials) was performed to investigate long-term memory performance. Memory errors were quantified according to Jarrard et al.'s definition [23]. The whole experiments was automatically controlled and monitored by a computer with MAZESOFT Software (Version 8.1.9).

1.3.3. Cholinergic neurons

The cholinergic neurons located in the septum and nucleus basalis of Meynert (nbM; Fig. 1B) play an important role in cognition and memory and are severely impaired in AD [24]. For immunohistochemistry brains were removed and fixed in 4% paraformaldehyde. Then brains were frozen under CO_2 snow and sectioned into 60-μm slices with a cryostate (Leica Jung CM3000). Slices were immunohistochemically stained for the enzyme choline-acetyltransferase (ChAT) that serves as markers for cholinergic neurons. ChAT-positive neurons of the nucleus basalis of Meynert (nbM) between Bregma −0.7 to −3.1 were counted at a 40× magnification under the microscope.

1.3.4. Inflammation

Inflammation plays a role in both AD as well as in vaD [25]. In our studies levels of inflammatory markers (e.g. interleukine-1β, tumor-necrosis-factor-α, monocytochemotactic-protein-1, macrophage-inflammatory-protein-2) in the cortex were explored by multiplex ELISA (Fig. 1C) (Searchlight, Aushon Biosystems), as described recently by us [21]. Briefly, the brains were removed, the frontal cortex dissected and immediately frozen. Cortex tissue was homogenized in ice-cold sodium phosphate buffer with a protease inhibitor, centrifuged and subsequently the supernatant was added to the prespotted plates and incubated for 3 h. After washing the biotinylated antibody was added and incubated for 30 min. After being washed streptavidin-horseradish peroxidise reagent was added and the luminescent signal was detected by the Searchlight CCD imaging and analysis system. Sample values were calculated from the standard curve in a liner range.

1.3.5. Vascular disruptions

Vascular risk factors may induce leakage of the blood–brain barrier allowing certain substances to migrate into the brain. For immunohistochemistry brains were removed and were frozen under CO_2 snow and sectioned into 20-μm sections with a cryostate (Leica Jung CM3000). Blood–brain barrier integrity was assessed by immunohistochemical staining against rat immunoglobulin G (IgG), as described in detail by us [21]. IgG-positive spots in the cortex were counted under the microscope at a 40× magnification.

1.4. Effects of long-term moderate vascular risk factors in vivo in rats

Treatment with the vascular risk factors cholesterol, homocysteine and ethanol over months resulted in memory impairment, dysfunction of the cholinergic system, blood–brain barrier leakage and inflammation in adult Sprague Dawley rats.

1.5. Effects of hypercholesterolemia

Cholesterol may play a role in development of AD possibly by influencing APP processing which leads to enhanced levels of Aβ [26]. Indeed, inhibitors of cholesterol synthesis (statins) may have a protective effect against AD [27] and the C-terminal transmembrane domain C99 of APP specifically binds cholesterol and favors the amyloidogenic pathway in cells by promoting localization of C99 in lipid rafts [28,29]. We have shown that treatment of rats with a 5% cholesterol-rich diet for 5 months caused spatial memory deficits, a dysfunction of the cholinergic system, blood–brain barrier leakage and inflammation [20]. In the cortex of cholesterol-fed rats levels of APP, $A\beta_{(1-42)}$, as well as tau and phospho-tau 181 were significantly enhanced [10]. Thus, hypercholesterolemia in rats resembled some AD-like pathology and has been suggested to provide a good model to study AD development as well as cerebrovascular dysfunction [10].

1.6. Effects of hyperhomocysteinaemia

Elevated homocysteine plasma levels may enhance the risk for developing AD or vaD [30]. It is well established that hyperhomocysteinaemia induces memory and learning disabilities in several animal models [21,31]. We have shown that in adult Sprague Dawley rats hyperhomocysteinaemia for 5 months markedly affected spatial memory performance and the number of cholinergic neurons in the nbM [21]. In the cortex blood–brain barrier leakage was enhanced after 12 months homocysteine treatment, but inflammation was not induced [21]. Interestingly, hyperhomocysteinaemia in rats did not show an AD-like pathology, such as Aβ-plaques, tau-pathology or inflammation, but resulted in spatial memory impairment and dysfunction of the cholinergic system which might be initiated by blood–brain barrier impairment [21].

1.7. Effects of ethanol

Ethanol consumption might cause cerebrovascular diseases, such as stroke and vaD, and may induce cognitive decline and cholinergic dysfunction [10] and AD [32–34]. However, there is increasing evidence that moderate chronic ethanol has protective effects on AD development [35]. We have shown that long-term treatment (12 months) of adult Sprague Dawley rats with 20% ethanol in drinking water ad libitum resulted in cognitive decline, cholinergic dysfunction and blood–brain barrier leakage, but did not dramatically induce cortical inflammation [22]. In the cortex of ethanol treated rats only monocyte chemotactic-protein-1 (MCP-1) was enhanced suggesting that MCP-1 may take part in a signaling cascade deviating from its role as a pro-inflammatory cytokine [22]. MCP-1 is also secreted by activated microglia cells [36] and indeed, after moderate long-term ethanol treatment cortical microglia reactivity was enhanced in rats [22]. Interestingly, ethanol did not resemble an AD-like pathology, but rather suggested a protective effect against AD development [22]. In summary, the cerebrovascular risk factor ethanol may markedly contribute to some pathology of vaD, but did not resemble an AD-like neuropathology [22].

1.8. Combination of vascular risk factors

The combination of ethanol and cholesterol did not dramatically affect the changes in spatial memory, cholinergic neurons, blood–brain barrier impairment and inflammation [22]. However, ethanol counteracted some of the cholesterol-induced effects: it reduced weight, plasma cholesterol levels and cortical $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ content, suggesting a protective role of ethanol in the development of AD [22]. In contrast, cholesterol did not markedly affect the ethanol-induced changes showing only a prominent reduction of plasma ethanol levels [22]. Cholesterol may modulate the absorption of ethanol into the blood, possibly due to a prolonged retention of ethanol in the stomach after cholesterol-rich nutrition [37]. Taken together, a combined treatment of ethanol and cholesterol did not potentiate the effects of a single treatment but rather counteracted some of the ethanol- or cholesterol-induced effects [22]. A combined cholesterol and homocysteine diet for 5 months resulted in spatial impairment and reduced cholinergic neurons similar to a single treatment, but homocysteine counteracted the cholesterol-induced inflammation and reduced slightly cortical blood–brain barrier leakage.

1.9. Long-term versus short-term effects: differences

Short-term (5 months) 5% cholesterol diet markedly reduced spatial memory performance, the number of cholinergic neurons in the nbM, induced inflammation as well as blood–brain barrier leakage in the cortex [20]. Interestingly, chronic ethanol or cholesterol treatment for 12 months had similar effects in vivo, although they were not as pronounced as the 5-month cholesterol effects. Long-term treatment of cholesterol did not result in inflammation and the changes were not as pronounced, most likely due to adaptive and compensatory mechanisms. This is in line with Pirchl et al. [21], who reported that short-term (5 months) treatment with the vascular risk factor homocysteine had more severe effects than long-term (15 months) homocysteine treatment. The cholinergic impairment after prolonged exposure to homocysteine was possibly counteracted by the upregulation of nerve growth factor [21], which is the most potent trophic molecule to support survival of cholinergic neurons [38].

2. Conclusion

Moderate chronic vascular risk factors, such as cholesterol, homocysteine or ethanol impair spatial memory, decline cholinergic neurons and induce blood–brain barrier leakage in rats in vivo which may contribute to the development of vascular dementia or Alzheimer's disease (Table 1).

Conflict of interest

None.

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Table 1
Effects of vascular risk factors in Sprague Dawley rats in vivo.

Risk factor	Spatial memory	Cholinergic neurons	Inflammation	Blood–brain barrier leakage
Cholesterol	↓	↓	↑	↑
Homocysteine	↓	↓	–	↑
Ethanol	↓	↓	–	↑
Ethanol + cholesterol	↓	↓	–	↑
Homocysteine + cholesterol	↓	↓		↑

Spatial memory was tested on the eight arm radial maze. The number of cholinergic neurons was measured by immunohistochemistry. Inflammatory markers were analyzed by multiplex Searchlight ELISA. Blood–brain barrier leakage was indirectly shown by anti-rat immunoglobulin G histochemistry. (↓ decreased, ↑ increased, – no change).

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